Original Article

Practical Guidelines for the High-Sensitivity Detection and Monitoring of Paroxysmal Nocturnal Hemoglobinuria Clones by Flow Cytometry

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Background: Paroxysmal nocturnal hemoglobinuria (PNH) is a life-threatening disorder caused by an inability to make glyco-phosphatidyl-inositol (GPI) anchors. While flow cytometry is the method of choice to detect the loss of GPI-linked proteins, the development and validation of sensitive, standardized, methodologies have been hampered by the rarity of this disease and by technical difficulties in the accurate identification of PNH cells.

Methods: Guidelines for the diagnosis and monitoring of PNH by flow cytometry were recently published by the International Clinical Cytometry Society (ICCS). However, specific reagent cocktails, and associated detailed analytic strategies were not directly addressed therein. In this supporting document based on the ICCS guidelines, we provide concise practical protocols for the high-sensitivity detection of PNH RBCs and WBCs (both granulocytes and monocytes).

Results: The CD235aFITC/CD59PE assay described was capable of detecting as few as 20 Type III PNH RBCs per million cells. Frequencies of Type III PNH cells in 10 normal samples were in the 0–6 per million RBCs. The high-resolution granulocyte/neutrophil assays described in this study could detect PNH phenotypes consistently at a level of 0.01% sensitivity. Frequencies of PNH phenotypes in normal individuals were in the 0–10 per million granulocytes/neutrophils range.

Conclusions: The careful screening and selection of specific antibody conjugates has allowed the development of reagent cocktails suitable for high-sensitivity flow cytometric detection of PNH RBCs and PNH WBCs. The reagent cocktails described herein can be used on a variety of clinical flow cytometers equipped with four or more photo multiplier tubes. © **2012 International Clinical Cytometry Society**

Key terms: high-sensitivity flow cytometry; paroxysmal nocturnal hemoglobinuria; practical guidelines

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare hematopoietic stem cell disorder resulting from the somatic mutation of the X-linked phosphatidylinositol glycan complementation Class A (*PIG-A*) gene (1–5). In normal individuals, this gene encodes an enzyme involved in the first stage of glyco-phosphatidyl-inositol (GPI) biosynthesis. In PNH, there is a partial or absolute inability to make GPI-anchored proteins including complement-defense structures such as CD55 and CD59 on RBCs and WBCs (6–9).

Additional Supporting Information may be found in the online version of this article.

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Clinical features of PNH include intravascular hemolysis (that leads to hemoglobinuria), bone marrow failure, and thrombosis, all major causes of morbidity and mortality (10,11). Recently, small PNH clones have been reported in patients with early stage myelodysplastic syndrome (particularly the refractory cytopenias with unilineage dysplasia variant). Preliminary results from a large study of patients with aplastic anemia (AA), myelodysplastic syndromes (MDS), and other bone marrow failure syndromes (EXPLORE) showed a significant number of MDS patients to have rare PNH phenotypes (12). However, more recent studies performed with high sensitivity flow methodologies have found much lower incidences of PNH clones in MDS (13,14).

There is a well-documented relationship between AA and PNH, with up to 40% of AA patients having PNH clones, depending on the level of assay sensitivity. Some reports suggest that PNH+ AA patients respond to immunosuppressive therapy (15,16) and other studies show 10%–25% of the latter will exhibit a PNH clonal expansion and progress to clinical PNH (17).

Although the ability to rapidly detect GPI-deficient cells by flow cytometry (18,19) has led to improved diagnosis (20), patient management, and prognosis in PNH and related disorders, many laboratories still use "routine" CD55- and/or CD59-based approaches that are neither accurate nor sensitive below the 1%–2% clone size, rendering them inadequate to detect small PNH clones in most PNH+ AA and MDS cases (21). While a variety of more sensitive approaches have more recently been developed for PNH WBC detection based on the bacterial lysin FLAER (22–26), FLAER-based assays, whether alone or cocktailed with other antibodies are still not widely deployed and recent data from External Quality Assurance programs have highlighted the variable capabilities of laboratories to accurately detect WBC PNH clones in stabilized whole blood samples (27,28).

Over the last few years, a humanized monoclonal antibody against the terminal complement protein C5 (29) (Eculizumab; Alexion, Cheshire, CT) has been approved for the treatment of patients with hemolytic PNH. This drug significantly reduces hemolysis, transfusion requirements, and thrombosis, and has improved the quality of life for PNH patients (30,31). Therefore, accurate detection, monitoring, and diagnosis of PNH have become increasingly important priorities for clinical flow laboratories performing PNH screening.

To address these issues, the International Clinical Cytometry Society (ICCS) recently published Guidelines for the Diagnosis and Monitoring of PNH and related disorders by Flow Cytometry (32). A variety of approaches for "routine" and "high-sensitivity" analyses (required in cases of MDS or AA) were outlined therein for both red blood cell and white blood cell lineages. Standardized Operating Protocols utilizing specific assay cocktails were not identified however.

For high-sensitivity red cell analysis in particular, where a CD235a and CD59 combination is preferred, it has been problematic to identify conjugates that in combination do not cause major aggregation of red cells while still maintaining a good signal-to-noise ratio and the ability to adequately separate Type II and Type III PNH red blood cells from normal (Type I) cells (22).

The goal of this study was to develop methods that are simple, easy to implement on a variety of instrument platforms and have uncomplicated data analysis. Particular attention was paid to sample preparation steps, selection of antibody clones and conjugates that give the highest signal-to-noise ratio. For the red cell assay, steps to limit RBC aggregates that are a significant issue with the use of CD235a conjugates were addressed. We have tested a variety of CD235a and CD59 clones/conjugates from several vendors leading to recommendations to optimize and standardize a two-color flow assay for the detection of PNH RBCs. For PNH WBC analysis, cocktails based on FLAER (an Alexa488 conjugate of Proaerolysin) were recommended by the authors of the ICCS Guidelines (32). We have thus developed a four-color combination for detection of PNH neutrophils using FLAER, CD24, CD15, and CD45. Similarly, a four-color combination of FLAER, CD14, CD64, and CD45 has been developed for the detection of PNH monocytes. Both assays can be deployed on a variety of clinical cytometers. The widespread adoption of highly standardized methodologies, coupled with improvements in quality control material to assess laboratory performance, would be expected to result in improved detection and monitoring of PNH clones in clinical flow laboratories.

MATERIALS AND METHODS

Antibody Clone/Conjugate Selection for High-Sensitivity PNH RBC Detection

Red blood cell gating antibodies. CD235a (Glycophorin A) is currently the only gating reagent available that specifically identifies mature RBCs. Preliminary screening of CD235a FITC and PE conjugates (clone KC16, Beckman Coulter) had shown the PE conjugate to cause far more aggregation of RBCs even after extensive titration than its FITC-labeled counterpart (22). In this study, we screened a number of other CD235a antibody conjugates for this assay including clones JC159 (Dako, Glostrup, Denmark), GA-R2 (BD Biosciences, San Jose, CA), NAM10-6G4 (IQ Products, Groningen, The Netherlands), and HIR2/GA-R2 (eBioscience, San Diego, CA).

GPI-specific antibodies. While loss of the GPI-linked CD55 and CD59 structures has traditionally been used to detect PNH RBCs (18,19), CD55 is inferior to CD59 (32). Therefore, in this study, we focused on identifying CD59 conjugates that offered the best separation of Type I, Type II, and Type III RBCs. CD59FITC (Clone P282) was obtained from Beckman Coulter. CD59FITC and CD59PE (clone P282) were obtained from BD Biosciences. CD59PE (clone MEM43) was obtained from

Invitrogen (Carlsbad, CA). CD59PE (clones MEM43 and OV9A2) were obtained from eBioscience.

As CD235a antibodies in particular are known to cause RBC aggregation, all CD235a and CD59 antibody conjugates were individually titrated against RBCs from normal individuals to minimize this effect while still providing for adequate separation of Type I, II, and III cells. It should be noted that all CD235a conjugates and the CD59PE conjugates of MEM43 and OV9A2 required considerable dilution below saturating levels to obviate this problem. Once optimally titrated, individual CD235a and CD59 conjugates were admixed in two-color combinations and where necessary, titrated again. As shown below, combinations of CD235aFITC (clone KC16)/CD59PE (Clone MEM43 or OV9A2) proved to be the best combination for delineating Type I, II, and III PNH populations in all samples tested.

Instrument Set-Up Considerations: RBCs

For light scatter and photo multiplier tubes (PMT) voltage set-up, a normal blood sample was diluted 1 : 100 with clean PBS. Forward angle (FS) and side angle (SS) light scatter voltages were established in log:log format such that the cluster of unstained normal RBCs could be identified towards the middle of the bivariate histogram (Fig. 1A, plot 1). When voltages are set in this manner, the presence of red cell aggregates and other debris can be readily addressed (see below).

For setting the FL1 and FL2 PMT voltages, all compensation was set to "zero" and the PMT voltages were established without the use of "baseline offset" for instruments so equipped. Gated RBCs from region R1 of plot 1 were displayed on an FL1 versus FL2 plot in log:log format and the PMT voltages adjusted so that the cells were comfortably on-scale (Fig. 1A, plot 2). Two-color compensation adjustments were performed with samples individually stained with CD235aFITC (Fig. 1A, upper right plot) and CD59PE (Fig. 1A, lower right plot). Instrument set-up was verified by analyzing a fresh normal sample stained as outlined below with the optimized CD235a/CD59 antibody cocktail (Fig. 1B, plots 1–3). If the voltage setting needed to be changed at a later point, this compensation process was repeated.

RBC Staining Procedure Using CD235aFITC/CD59PE Cocktail

Peripheral blood samples less than 48 h old (anti-coagulated with EDTA) were diluted 1:100 with phosphate-buffered saline. Fifty microliters of diluted blood sample was pipetted into a test tube using reverse-pipetting to avoid aerosol generation.

Based on individual titrations of single lots of CD235a-FITC and CD59PE, a cocktail of these reagents was prepared. Fifteen microliters (1.5 μL/test) of CD235aFITC (clone KC16 Beckman Coulter) and 5 μL (0.5 μL/test) of CD59PE (clone MEM43 Invitrogen) were diluted with 180 μL PBS and 20 μL of this diluted cocktail was added directly into the blood sample in the test tube with gently mixing by up-and-down pipetting. (Note: more recent lots of CD235aFITC have shown a requirement for 5 μL/test). After careful removal of the pipette tip, the sample was further mixed by gently swirling the sample using a slow speed vortex, again taking care not to generate aerosols.

The sample was incubated in the dark for 20 min at room temperature (incubation times of up to 60 min generated identical results), washed twice with PBS by centrifugation as is required to optimize separation of Type I, II, and III red blood cells (20,32) and re-suspended in 0.5–1.0 mL of PBS. The sample was "racked" (dragged vigorously across a hard plastic or metal test tube rack several times) to disrupt any RBC aggregates generated by the staining/washing procedure immediately before acquisition on the cytometer. Samples were generally acquired immediately, as delays longer than 15 min after the final washing step typically show decreased CD235a staining in the hands of the authors.

A minimum of 100,000 RBCs (gated on log FS vs. log SS) were acquired in list mode for clinical test samples. If two or more events were displayed in the Type III PNH RBC region data acquisition was continued until 1 million events were acquired.

Antibody Clone/Conjugate Selection for High-Sensitivity PNH WBC Detection

Gating reagents for granulocytes and monocytes. In developing highly sensitive assays for granulocytes and monocytes, the ICCS Guidelines (32) stressed the need to use an antibody for gating each lineage as well as two GPI-specific markers for each lineage. While CD33 can be used to gate both granulocytes and monocytes (22,25), this reagent is not as effective as CD15 for delineating granulocytes or as effective as CD64 for delineating monocytes (17; D.R.S., A.I., M.K., unpublished data). Consequently, conjugates of CD15 (for granulocytes) and CD64 (for monocytes) were selected for this study.

GPI-specific reagents. Although CD55 and CD59 have been widely deployed due to their use in the earliest flow-based assays to detect PNH (18–20), recent studies using stabilized whole blood have shown that these reagents are not optimal for the detection of PNH granulocytes and monocytes (27). While the ICCS Guidelines identified CD24, CD16, and CD66b along with FLAER as the most reliable reagents for PNH granulocyte detection, we selected CD24 and FLAER as GPI-specific reagents for granulocyte assessment and CD14 and FLAER as GPI-specific reagents for monocyte assessment (for rationale, see Supporting Data).

Specifically for this study, the granulocyte cocktail contained FLAER and CD45PECy7 (clone J33) together with the granulocyte-gating reagent CD15PECy5 (clone 80H5) and a granulocyte-associated GPI-specific reagent CD24PE (clone ALB9). The monocyte cocktail contained, in addition to FLAER and CD45PECy7, a monocyte-gating reagent CD64PECy5 (clone 22), and a monocyte associated GPI-specific reagent CD14PE (clone RMO52) (all antibodies from Beckman Coulter; FLAER was purchased from Cedarlane).

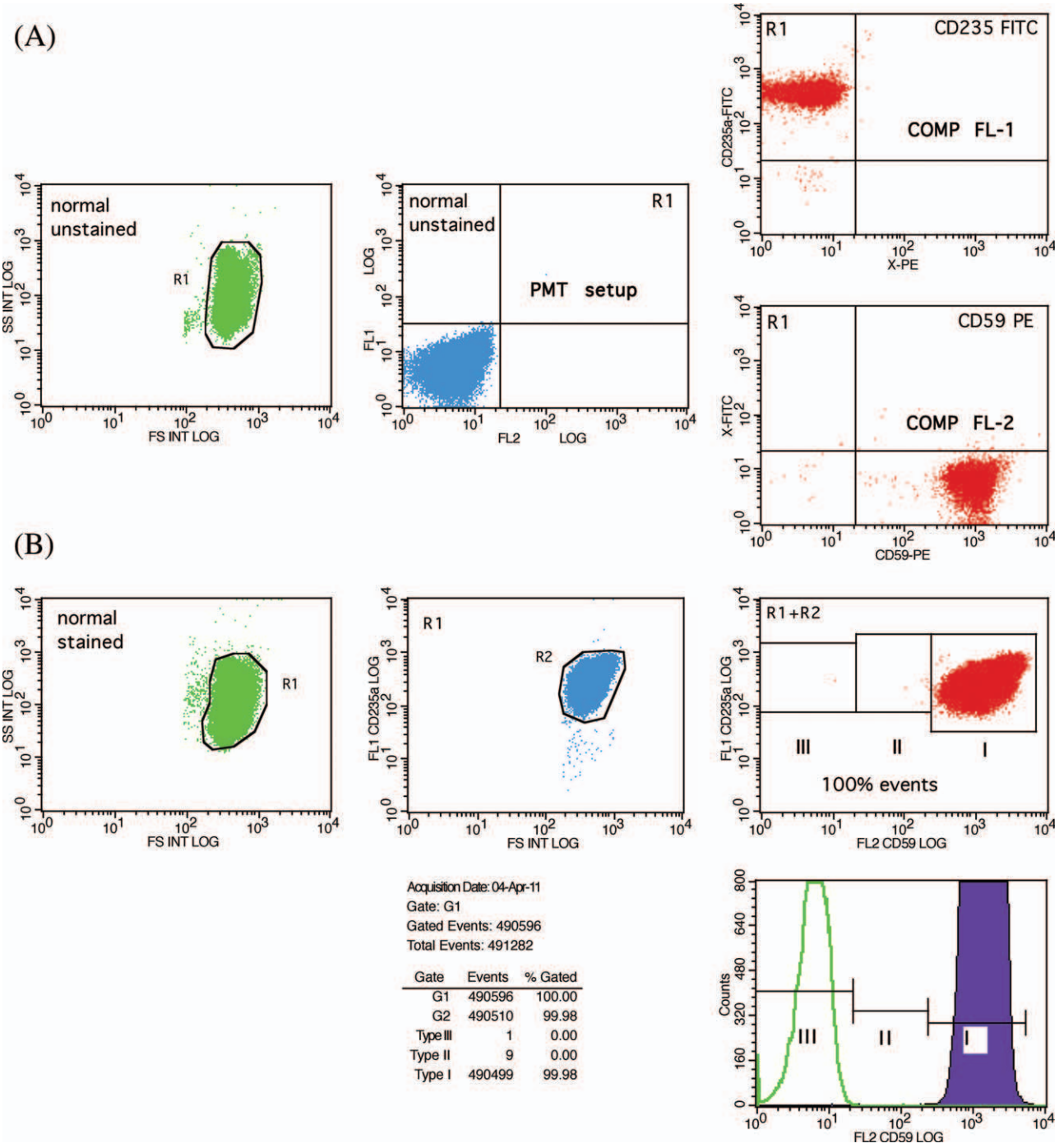

FIG. 1. (**A**) Instrument set-up for high-sensitivity RBC assay. Light scatter voltages were established in logarithmic mode such that RBCs from a diluted normal PB sample clustered in the middle of the plot (left). Gated RBCs from Region R1 were displayed on FL1 versus FL2 plot and PMT voltages adjusted to get the unstained RBCs properly on-scale (middle). Samples were single stained with either CD235aFITC (top right) or CD59PE (bottom right) and compensation adjusted to reduce spectral overlap. (**B**) Analysis of normal RBCs. Normal RBCs stained using the instrument settings established in (A). RBCs gated in R1 (left), and displayed on CD235aFITC versus FS plot (middle). CD235a+ RBCs were gated in R2 and cells from R1 and R2 displayed on CD235aFITC versus CD59PE. Normal Type I RBCs were gated as shown in gate I. A second sample stained only with CD235aFITC was also analyzed to mimic the location of the Type III gate (gate III). (**C**) Analysis of fresh PNH sample. A PNH sample was stained as described with the optimized RBC protocol as outlined in the Methods, and analyzed as described for the normal sample (B). The sample contained 42.41% PNH Type III cells and 5.89% PNH Type II cells (statistics obtained from top right dot plot).

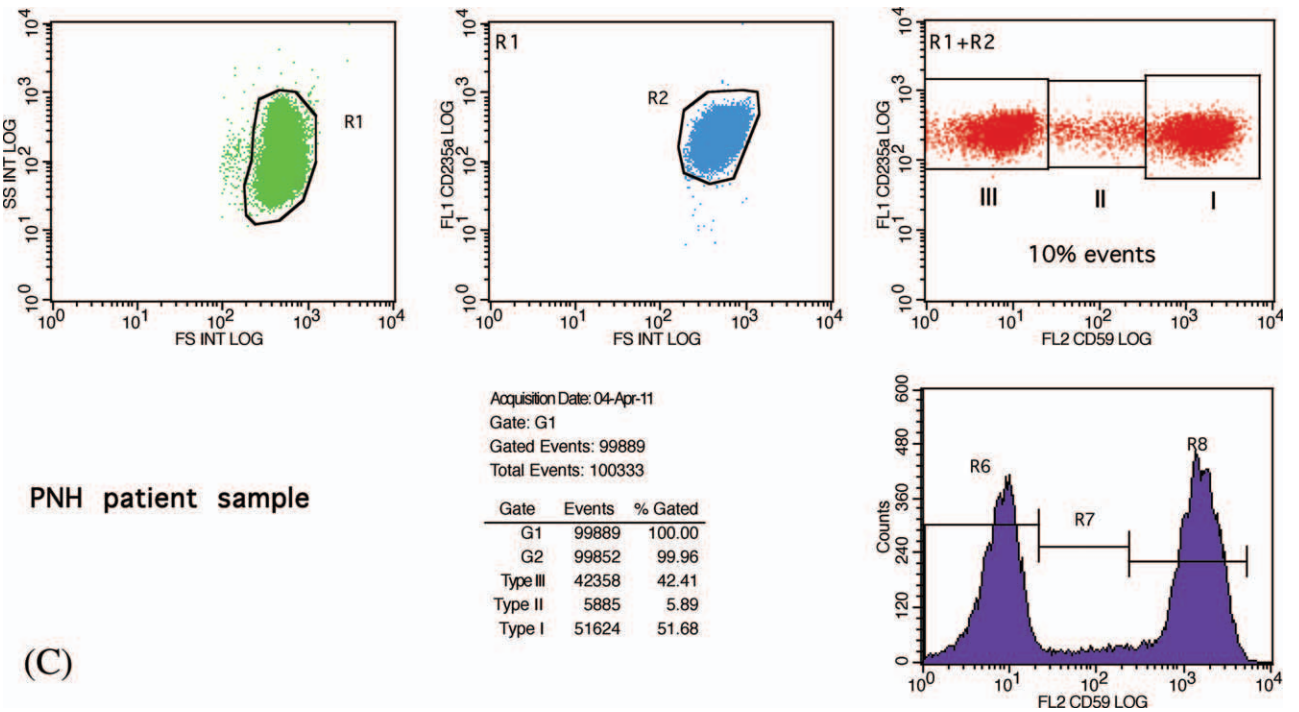

FIG. 1. continued

In some experiments performed on the FACSCalibur instrument (BD Biosciences), CD15 PerCP:Cy5.5 (clone HI98, BD Biosciences) was used in place of CD15PECy5 and CD45APC (Clone 2DI) was used in place of CD45PECy7 (much greater compensation is required between the FL3 and FL4 channels of the FACSCalibur when PECy5 and APC conjugates are used together, in comparison with that required when PECy5.5 conjugates are used with APC-conjugated antibodies).

Instrument Set-Up Considerations: WBCs

For light scatter and PMT voltage set-up for WBC, 100 μL of a washed, lysed, unstained normal blood sample was used (Figs. 2 and 3). FS and SS light scatter voltages were established in lin:lin format and the voltages adjusted such that all major leukocyte subsets including lymphocytes were clearly visible above the FS threshold (Fig. 2, plot 1 and Fig. 3 plot 1 for granulocyte and monocyte assays, respectively). These settings allow for the identification of cells with PNH phenotype within granulocyte and monocyte lineages and for the targeting of lymphocytes representing "internal" positive and negative reagent controls.

For setting the PMT voltages, all compensation was set to "zero" and the PMT voltages were established without the use of "baseline offset" for instruments so equipped. Because of the different emission spectra of Alexa488 and FITC, compensation was established using a FLAERAlexa488 stained sample in the FL1 channel. Thus, the FL1 PMT voltage was established using a PNH sample that contained some PNH lymphocytes. The sample was single-stained with fluorescent Proaerolysin (FLAERAlexa488) (liquid form, Cedarlane Labs, Burlington, ON, Canada), and the FL1 PMT voltage was adjusted so that PNH lymphocytes were comfortably on scale. A second sample was stained with CD3PE and the FL2 PMT voltage was adjusted to ensure that non-T lymphocytes were also comfortably on scale. This procedure was repeated using CD3PECy5 and CD3PECy7 to set the FL4 and FL5 PMT voltages of the FC500 cytometer. These voltages were used for the compensation process. Compensation adjustments on the FC500 were made using blood samples individually stained with FLAER Alexa488 (FL1), or CD45PE (FL2), or CD45PECy5 (FL4) or CD45PECy7 (FL5). Instrument settings were optimized and verified by analyzing the PNH sample stained with a FLAERAlexa488, CD24PE, CD15PECy5, and CD45PECy7 cocktail. As per RBC assay development, all PMT voltage and compensation settings were established without the use of the baseline offset facility on instruments so-equipped. If PMT voltages appeared to be suboptimal after compensation and required adjustment, the compensation process was repeated.

WBC Staining Procedure for Optimized Granulocyte and Monocyte Cocktails

All individual antibodies were verified for appropriate reactivity with target cells and titrated to optimize specific staining performance prior to being cocktailed for use in the high-sensitivity FLAER-based granulocyte and monocyte assays. One hundred microliters of fresh

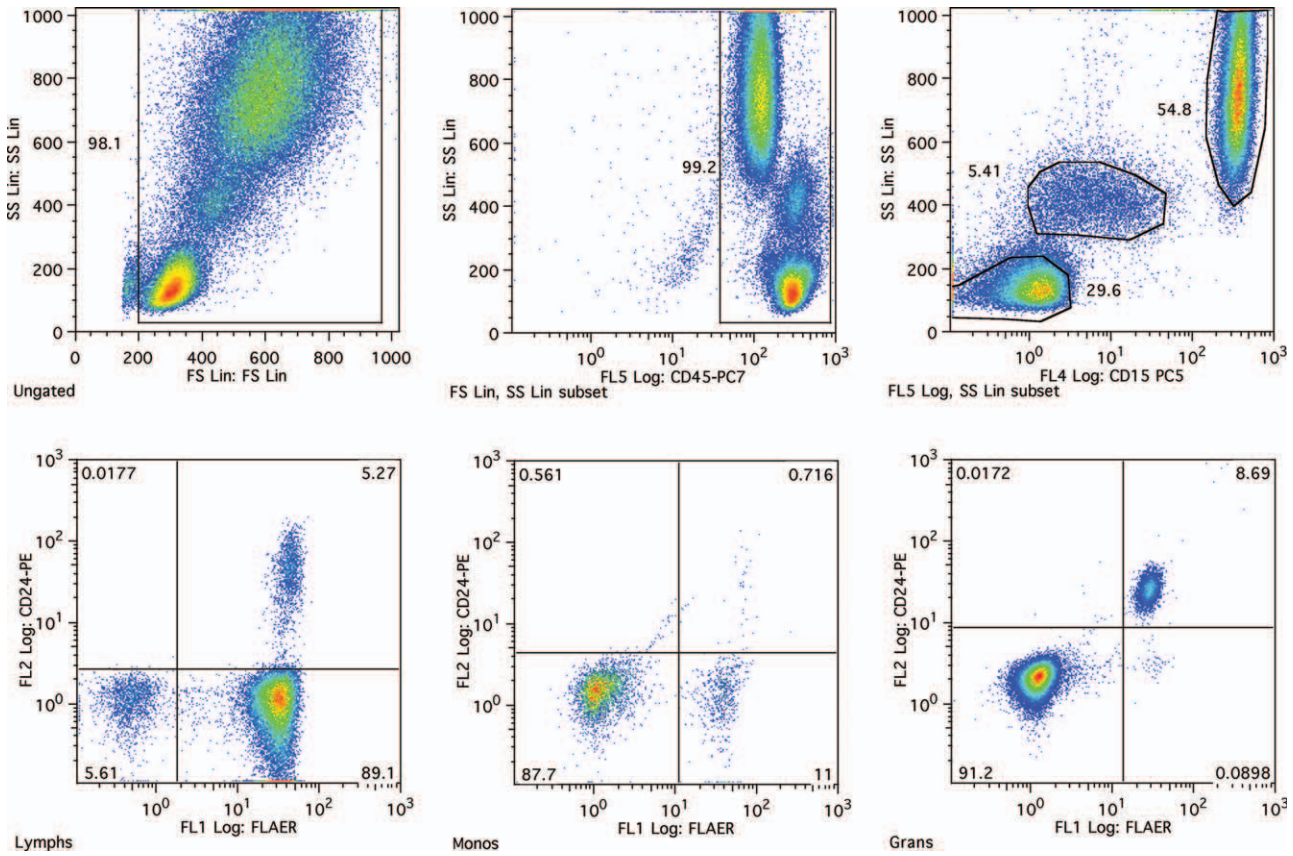

FIG. 2. Example of high-sensitivity granulocyte assay. A sample from a long-term PNH patient was stained with FLAER, CD24PE, CD15PECy5, and CD45PECy7. Light scatter voltages were established so that all nucleated cells were visible above the forward scatter threshold (top left) as described in the Methods section, and debris excluded with a combination of light scatter and CD45 gating (top middle). CD45+ events were displayed on CD15 versus SS plot (top right) and granulocytes (bright CD15, high SS), monocytes (dull CD15 and intermediate SS), and lymphocytes (CD15-negative, low SS) gated. Each of these populations was displayed on a FLAER versus CD24 plot (bottom row). PNH granulocytes (FLAER-negative, CD24-negative) were enumerated in the bottom right plot (lower left quadrant). Normal granulocytes were enumerated in the upper right quadrant. Gated monocytes were similarly displayed (bottom row middle) and the PNH monocytes (FLAER-negative, CD24-negative) were enumerated in the lower left quadrant. Gated lymphocytes (bottom row left) were assessed for PNH phenotypes in the lower left quadrant. Normal T lymphocytes (FLAER+, CD24-negative) are visible in the lower right quadrant and normal B lymphocytes (FLAER+, CD24+) are visible on the upper right quadrant.

peripheral blood was carefully pipetted into the bottom of a test tube without touching the side of the tube. Although blood samples have been shown to be stable for up to 7 days if kept cold (see Supporting Figure 11), the ICCS Guidelines recommended staining samples within the first 48 h from sample draw (32). An appropriate volume of "granulocyte" (FLAERAlexa488, CD24PE, CD15PECY5, and CD45PECy7) or "monocyte" (FLAERAlexa488, CD14PE, CD64PECy5, and CD45PECy7) cocktail was added directly to the blood aliquot in the bottom of the tube and mixed gently.

After a 20 min incubation period in the dark, the red blood cells were lysed. Immunoprep (Beckman Coulter), FACSLyse (BD Biosciences), Optilyse C (Beckman Coulter), and Ammonium chloride-based lysing agents are all acceptable lysing agents, although those containing fixatives may help retain cellular integrity better than those that do not. After lysing, cells were washed once with PBS, resuspended in 1 mL of PBS and acquired on the cytometer.

Verification and Validation of High-Sensitivity RBC Assay

Frequencies of cells with PNH phenotype in normal samples. The frequency of cells with a Type III PNH RBC phenotype in 10 normal samples was determined using the optimized RBC assay described earlier. Data were acquired for 1 million RBCs.

RBC assay sensitivity. Ten microliters of accurately pipetted PNH blood was carefully mixed with 100 μL of normal blood. Ten microliters of this first dilution was accurately removed and admixed as above in a second tube that also contained 100 μL of normal blood. The same process was repeated a further three times until a range from 1:10 to 1:10,000 was established. Samples at each dilution (as well as an undiluted sample) were stained as above with the optimized RBC assay. Up to 1

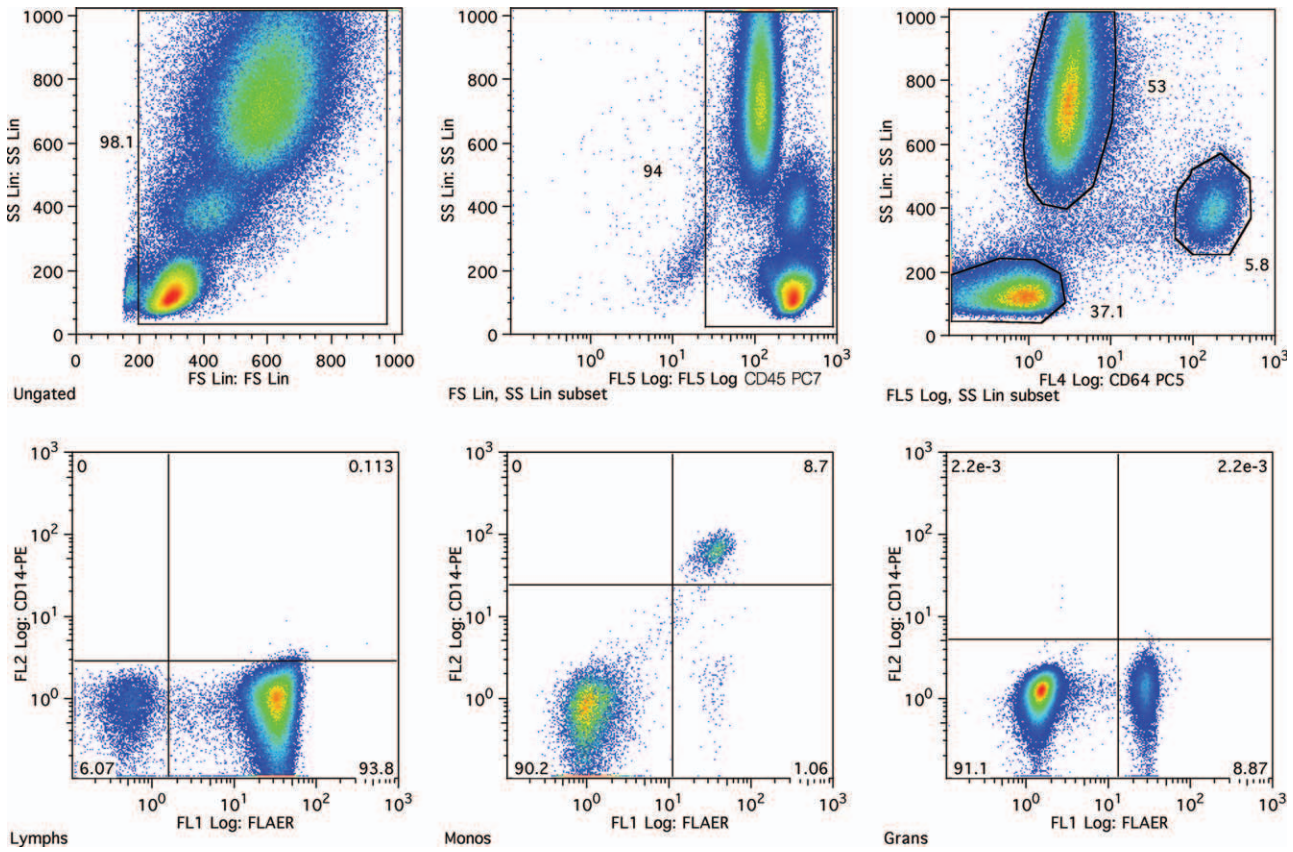

FIG. 3. Example of high-sensitivity monocyte assay. A sample from a long-term PNH patient was stained with FLAER, CD14PE, CD64PECy5, and CD45PECy7 and analyzed in a similar manner to that shown for the granulocyte assay in Figure 2. CD45+ events were displayed on CD64 versus SS plot (top right) and granulocytes (dull CD64, high SS) monocytes (bright CD64, intermediate SS) and lymphocytes (CD64-negative, low SS) were gated. Each of these populations was displayed on a FLAER versus CD14 plot (bottom row). PNH granulocytes (FLAER-negative, CD14-negative) were enumerated in the bottom right plot (lower left quadrant). Normal granulocytes were enumerated in the lower right quadrant. Gated monocytes were similarly displayed (bottom row middle) and the PNH monocytes (FLAER-negative, CD14-negative) were enumerated in the lower left quadrant. Gated lymphocytes (bottom row left) were assessed for PNH phenotypes in the lower left quadrant. Normal lymphocytes (FLAER+, CD14-negative) clustered in the lower right quadrant.

million events were collected in list mode at the 1:1,000 and 1:10,000 dilutions.

Verification and Validation of High-Sensitivity Granulocyte and Monocyte Assays

Frequencies of cells with PNH phenotype in normal samples. The frequency of cells with PNH phenotype among granulocytes in 10 normal samples was determined using the optimized FLAER-CD24-CD15-CD45 granulocyte assay described earlier. Data were acquired for 100,000 CD15-gated granulocytes and the number of FLAER-negative/CD24-negative events was recorded from the dot plot. A similar experiment was performed to determine the frequency of cells with PNH phenotype in monocytes in 10 normal samples using the optimized FLAER-CD14-CD64-CD45 monocyte assay described earlier. Data were acquired until 20,000 CD64-gated monocytes were acquired or for a maximum of 10 min and the number of FLAER-negative/CD14-negative events was recorded.

WBC Assay sensitivity. A fresh PNH sample was diluted serially 1:10, 1:100, 1:1,000, and 1:10,000 with a normal blood sample as described earlier to establish WBC assay sensitivity. At least 100 FLAER-negative/CD24-negative PNH granulocytes were collected except at greatest dilutions at which data were collected for a maximum of 10 min per tube. A similar experiment was performed to determine the sensitivity of the monocyte assay. While it was relatively easy to collect at least 100 FLAER-negative/CD14-negative PNH monocytes at the 1:10 and 1:100 dilutions, the 1:1,000 and 1:10,000 tubes were terminated after the maximum 10 min acquisition time.

RESULTS

High-Sensitivity RBC Assay

In the experiment shown in Figure 1B, RBCs from a normal blood sample were stained using the optimized staining protocol described earlier. After two washes and racking of the sample immediately prior to data acquisition, the level of aggregates present in this analysis was under 1% of the total RBCs acquired in the data file. The frequency of events with Type III RBC phenotype

present in this analysis was 1 in 490,000 RBC events collected.

We next stained a known PNH sample from a patient receiving the therapeutic anti-C5 monoclonal antibody eculizumab (Soliris, Alexion Pharmaceuticals, Cheshire, CT) (Fig. 1C). Using the optimized RBC assay described earlier, this sample was shown to contain 42.4% Type III and 5.9% Type II RBCs. These data were derived from the bivariate CD235aFITC (KC16)/CD59PE (MEM43) plot displaying only 10% of the acquired events. This approach, or the use of density plots, allows for the most accurate setting of the Type I, II, and III gating regions (particularly on samples with low frequencies of PNH phenotypes) and is the recommended method rather than the single parameter histogram shown for comparison on the bottom row.

Assessment of PNH RBC Frequencies in Normal Samples

Ten normal blood samples were stained in each of the authors' laboratories with the optimized high-sensitivity RBC assay, and 1 million gated RBCs were acquired. The number of Type III RBCs detected ranged from 0 to 6 per million (mean 4 per million).

Sensitivity of the RBC Assay

As shown in Table 1, the undiluted PNH sample contained 34.86% PNH Type III RBCs. After the first 1:10 dilution, the PNH clone size detected was 1.77%. This result is explained by the different hematocrits of the PNH and normal blood samples. Expected numbers of PNH phenotypes were detected at subsequent dilutions and it was possible to detect 20 clustered Type III events in a total of 1 million RBCs at the 1:10,000 dilution. A minimum of 100 Type III RBCs were collected at every dilution except 1:10,000 where data were acquired until 1 million gated RBCs were collected.

Table 1
Two-Color Red Blood Cell Assay Sensitivity

Dilution	Type III RBCs	Sensitivity (%)
Undiluted	348,626	34.86
1:10	17,665	1.77
1:100	1,822	0.18
1:1,000	203	0.02
1:10,000	20	0.002

High-Sensitivity WBC Assay for Granulocytes

A four-color protocol for PNH sample analysis was established on an FC500 instrument equipped with 5 PMTs and a single 488 laser as described in the Methods section and stained with FLAERAlexa488, CD24PE, CD15PECy5, and CD45PECy7. The FCS3.0 data file was analyzed using FlowJo software (v9.3.1, Treestar Inc.).

As shown in Figure 2 for the granulocyte assay, all nucleated WBCs were gated to exclude debris (top row left). The gated WBCs were then displayed on a CD45 versus SS plot (top row middle). Gated CD45+ events were then displayed on CD15 versus SS (top row right). Granulocytes (bright CD15 staining and high SS) were gated as well as the monocytes (CD15 dull, intermediate SS) and lymphocyte population (CD15-negative, SS low). Gated granulocytes were then displayed on a FLAER versus CD24 plot (bottom row, right) and quadrants established to delineate normal granulocytes (FLAER+, CD24+) from PNH granulocytes (FLAER-negative, CD24-negative). As shown, 91.2% of the gated granulocytes exhibit a PNH phenotype. The gated monocytes were also displayed on a bivariate FLAER versus CD24 plot (bottom middle) and quadrants established to delineate normal monocytes (FLAER+, CD24-negative) from PNH monocytes (FLAER-negative, CD24-negative). In the sample shown in Figure 2, 87.7% of the gated monocytes exhibited a PNH phenotype. Similarly, gated lymphocytes were also displayed on a bivariate FLAER versus CD24 plot (bottom left) and quadrants established to delineate normal B-cells (FLAER+, CD24+) from normal T-lymphocytes (FLAER+, CD24-negative) and PNH lymphocytes (FLAER-negative, CD24-negative). In this sample, 5.61% of the gated lymphocytes exhibited a PNH phenotype (lower left quadrant). The appearance of PNH granulocytes, monocytes and lymphocytes on-scale in both FLAER and CD24 parameters allows the operator to see that instrument set up and compensation settings for the four-color granulocyte assay are optimal.

High-Sensitivity WBC Assay for Monocytes

A similar gating strategy was established for the high-sensitivity monocyte assay (Fig. 3). Debris and non-WBCs were excluded by a combination of light scatter gating (top left) and CD45 versus SS gating (top middle) and the gated WBC displayed on a CD64 versus SS plot (top right). Monocytes were gated based on their high expression of CD64, while granulocytes were gated based on low CD64 and high SS characteristics and lymphocytes by their lack of staining with CD64 and low side scatter. Gated monocytes were then displayed on a FLAER versus CD14 plot (bottom row, middle) and quadrants established to delineate normal monocytes (FLAER+, CD14+) from PNH monocytes (FLAER-negative, CD14-negative). As shown, 90.2% of the gated monocytes exhibited a PNH phenotype.

The CD64-gated granulocytes were also displayed on a bivariate FLAER versus CD14 plot (bottom row, right) and quadrants established to delineate normal granulocytes (FLAER+, CD14-negative) from PNH granulocytes (FLAER-negative, CD14-negative). Ninety-one percent of the granulocytes exhibit a PNH phenotype, in close agreement to the data derived from the granulocyte tube (Fig. 2).

The gated lymphocytes were also displayed on a bivariate FLAER versus CD14 plot (bottom left) and quadrants established to delineate normal lymphocytes (FLAER+, CD14-negative) from PNH lymphocytes (FLAER-negative, CD14-negative). A total of 6.07% of the gated lymphocytes exhibited a PNH phenotype (lower left quadrant) in close agreement with the data for this subset from the granulocyte tube. As for the granulocyte

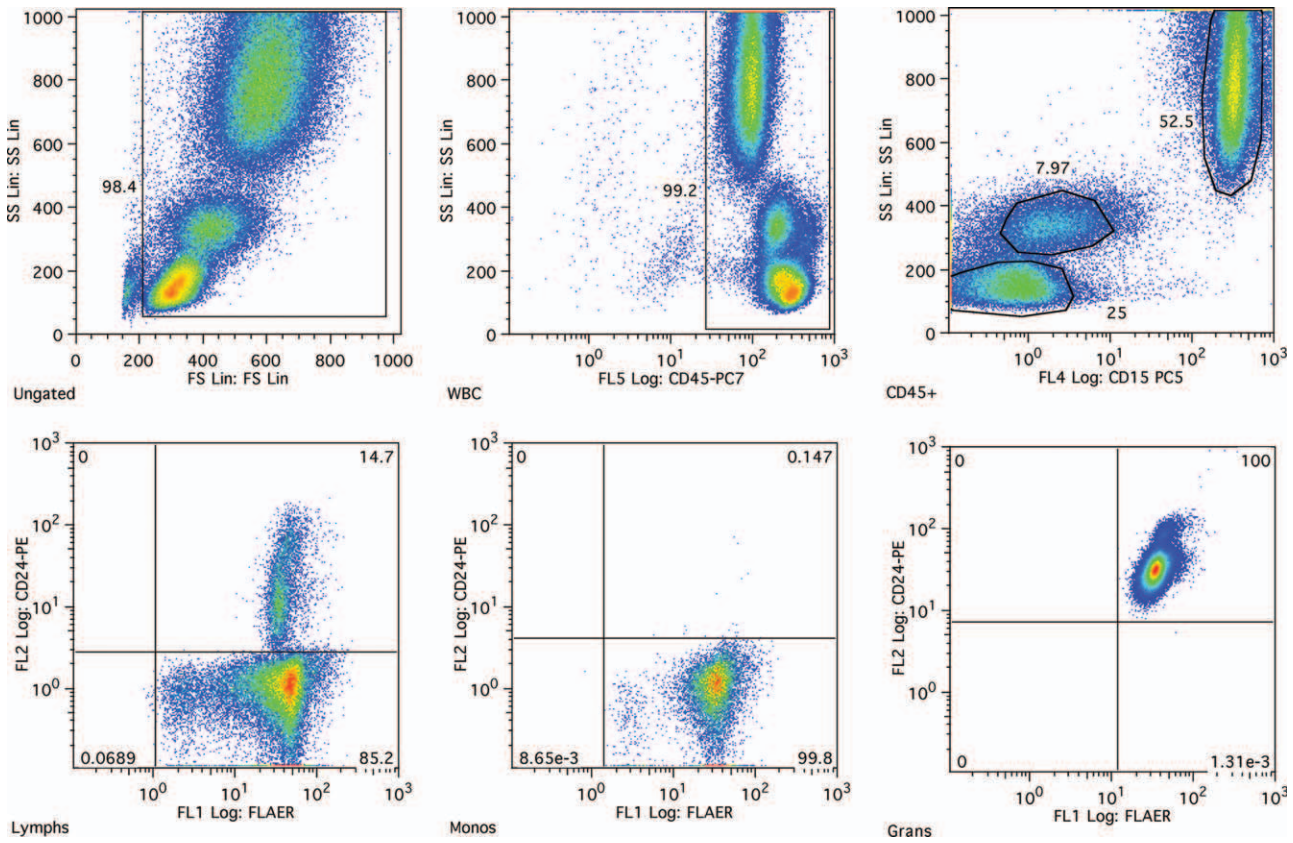

FIG. 4. Example of normal sample stained with high-sensitivity granulocyte assay. Normal sample stained and analyzed exactly as described in Figure 2. Zero PNH granulocyte phenotypes were detected in the lower left quadrant of the bottom right plot.

assay, the appearance of PNH granulocytes, monocytes, and lymphocytes on-scale in both FLAER and CD14 parameters allows the operator to see that instrument set up and compensation settings for the four-color monocyte assay are optimal.

Assessment of PNH Granulocyte and PNH Monocyte Frequencies in Normal Samples

Ten normal blood samples were stained with the high-sensitivity granulocyte cocktail as described in the Methods section. An example of a normal sample stained with the granulocyte cocktail is shown in Figure 4. A mean of 86,000 (range 61,000–113,000) CD15-gated granulocytes were acquired and PNH phenotypes accounted for a mean of 0.0013% over the 10 samples.

The same 10 normal samples were also stained with the high-sensitivity monocyte assay. An example of a normal sample stained with the monocyte cocktail is shown in Figure 5. A mean of 15,000 (range 6000–24,000) CD64-gated monocytes were acquired and PNH monocyte phenotypes accounted for a mean of 0.0033% (range) over the 10 samples assessed.

Sensitivity of the WBC Assay: Granulocytes

A fresh PNH sample containing approximately 91.3% PNH granulocytes was serially diluted with a normal blood sample at levels of 1:10, 1:100, 1:1,000, and 1:10,000. Comparing the undiluted PNH sample with the 1:10 diluted sample it can be inferred that the granulocyte counts of the PNH sample and normal sample used for the dilutions were similar. Up to 100,000 CD15-gated granulocytes were collected at the lower dilutions. The data shown in Table 2 indicate that this PNH granulocyte assay is sensitive to a level of 0.01%.

Sensitivity of the WBC Assay: Monocytes

The same PNH case containing approximately 89.8% PNH monocytes was serially diluted with a normal blood sample at levels of 1:10, 1:100, 1:1,000, and 1:10,000. Comparing the undiluted PNH sample with the 1:10 diluted sample, it can be inferred that the monocyte count of the PNH sample was approximately half that of the normal sample used for the dilutions. CD64-gated monocytes were collected at each dilution for up to 10 min per sample. The data shown in Table 3 indicate that this PNH monocyte assay is sensitive to a level of 0.04%. The lower sensitivity of the monocyte assay determined here likely reflects the fact that monocytes are generally found in smaller numbers than granulocytes in most samples.

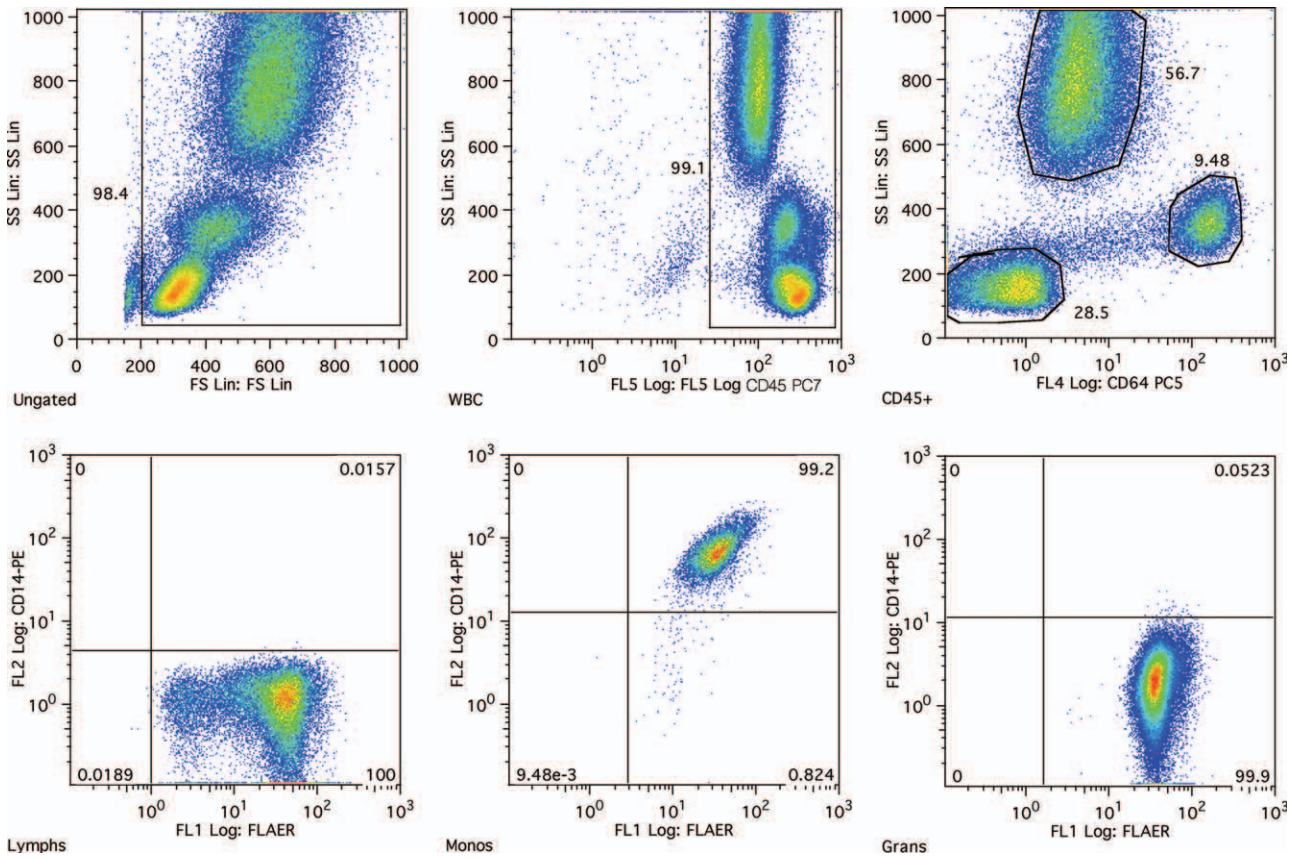

FIG. 5. Example of normal sample stained with the high-sensitivity monocyte assay. Normal sample stained and analyzed exactly as described in Figure 3. Only 1 PNH monocyte phenotype was detected in the lower left quadrant of the bottom middle plot.

DISCUSSION

In previous studies, we used a simple RBC assay based on light scatter gating and CD59 expression. The latter, while able to detect clinical PNH cases, did not provide sufficient sensitivity to detect clones less than 1%–2% (22). MDS and AA patient samples have been reported to contain very small PNH clones in the 0.01%–1% range (11,15,21,32). The previous method was also susceptible to false-negatives in which poorly stained samples would show evidence of PNH phenotypes that on re-staining would no longer be detectable (22).

In this manuscript, we detail a new assay for accurately detecting PNH RBCs at very high levels of sensitivity. In developing this assay, we screened a number of different CD235a and CD59 conjugated antibodies from a variety of vendors and titrated them both individually and in combination to identify an optimal "cocktail" for the assay (see Supporting Data).

Of the CD59 conjugates tested, the MEM43 and OV9A2 clones conjugated with phycoerythrin exhibited the best staining characteristics (signal-to-noise ratio) and allowed optimal separation between Type III and Type II PNH cells as well as separation from Type I (normal) cells while promoting minimal RBC agglutination. This was the case regardless of vendor source of the MEM43 conjugate. With respect to CD235a antibody conjugates, we had previously shown that PE-conjugated CD235a (clone KC16) caused significantly more RBC agglutination than its FITC counterpart (22). All screening, titration and testing of CD235a and CD59 conjugates performed for this study therefore utilized the

Table 2
Four-Color Granulocyte Assay Sensitivity

Dilution	PNH Granulocytes	Sensitivity (%)
Undiluted	51,420	91.3
1:10	5,799	9.4
1:100	573	0.94
1:1,000	91	0.089
1:10,000	9	0.01

Table 3
Four-Color Monocyte Assay Sensitivity

Dilution	PNH Monocytes	Sensitivity (%)
Undiluted	12,718	89.8
1:10	1227	4.1
1:100	112	0.4
1:1,000	13	0.044
1:10,000	ND	ND

"racking" technique to disperse any aggregates immediately prior to data acquisition as part of the sample preparation regimen. Most of the individual CD235a conjugates tested in this manner (after appropriate titration) were found to adequately stain RBCs without causing unacceptable levels of agglutination, although some variations were noted between different clones and conjugates. However, because the CD59PE conjugates of MEM43 and OV9A2 gave the best separation of PNH RBC subsets, we chose to find the best CD235a conjugates compatible with PE conjugated MEM43 or OV9A2, to minimize further testing. We performed most of our subsequent verification and validation studies with the simple FL1 and FL2 combinations of either KC16FITC/MEM43PE or KC16FITC/OV9A2PE.

The new RBC assay is highly sensitive, capable of detecting as few as 20 clustered Type III RBCs in "spiking" experiments. It also has a very low detection frequency of PNH phenotypes in normal samples (approximately four cells with Type III PNH phenotype per million). This method also has an advantage for labs performing infrequent testing in that FITC and PE conjugates have a longer shelf life than conjugates made with tandem dyes as they are not prone to breakdown of the tandem dye components (33).

While single parameter histograms have traditionally been used for enumerating Type I, Type II, and Type III cells, bivariate dot plots or density plots are more informative and their use is strongly recommended (see Supporting Data).

With respect to WBCs, while a variety of GPI-specific reagents such as CD55, CD59, CD16, CD66b, CD24, and CD14 have been widely used to detect PNH phenotypes (18–21), FLAER-based reagent cocktails that include lineage-specific gating reagents have become more widely deployed in recent years (22–26,32,34–36). We, and others have previously designed CD33-based assays to identify granulocyte and monocyte lineages in which the gated subsets can subsequently be assessed for FLAER and individual GPI-linked antigen expression (22,25). While FLAER-based assays have better sensitivity and accuracy over traditional CD55 and CD59-based assays, CD33-based gating of granulocytes and/or monocytes is sub-optimal in the four-color high-sensitivity setting. There are a number of reasons for this and are they are detailed in the Supporting Data.

For this study, we therefore designed a FLAER-based assay that utilized a combination of light scatter and CD45 expression to remove debris and exclude non-WBCs. CD45+ WBCs were then analyzed for CD15 expression to identify neutrophils. Gated neutrophils were then assessed for PNH phenotypes with a combination of CD24 and FLAER. A limited screen of CD15 clones showed some (e.g., clone 80H5) to be clearly superior to others in terms of optimally separating neutrophils from monocytes. With the substitution of the APC-conjugated form of CD45 for the CD45-PECy7 conjugate, the CD15-based assay generated essentially identical results on a FACSCalibur instrument when assessing the same samples on the same day (data not shown).

Because of the sensitivity of the CD15-based granulocyte assay (reliably detecting PNH phenotypes down to 0.01%), and the low frequency of PNH phenotypes in normal samples (0.0013% over 10 samples) this assay is capable of deployment in laboratories performing high-sensitivity analysis.

For PNH monocyte detection, FLAER and CD14 were recommended in the ICCS Guidelines. In this study, CD64 was used in preference to CD33 to gate monocytes as CD64 is not expressed on basophils, and primitive myeloid precursors. However, it should be emphasized that a CD64 clone such as clone 22 with optimal signal-to-noise ratio should be selected to minimize the presence of other cells in the monocyte gate.

Together with FLAER, CD14 was selected as a monocyte-specific GPI-linked structure. While CD14 reportedly is not expressed on dendritic cells (37), low level expression of CD14 on putative dendritic cells and/or monocyte precursors can be noted in some samples, within the CD64-gated monocyte population. However, normal dendritic cells and/or monocyte precursors should not be mistaken for PNH monocytes because they bind FLAER at the same level as normal monocytes. The high sensitivity monocyte assay has a very low frequency of PNH phenotypes in normal samples (less than 0.004%) and proved capable of high-sensitivity detection of PNH monocytes (0.04% or better). The monocyte assay may be as sensitive as the granulocyte assay but due to the numerically smaller monocyte populations in most samples, we were not able to test this as we were unable collect sufficient monocytes within the 10 minute acquisition time we allowed for data collection.

For laboratories setting up this assay that do not have access to a *bona fide* PNH sample, one way to check or verify compensation settings in the absence of a suitable PNH sample is to perform a "fluorescence-minus two" staining in which a normal sample is stained with only CD45 and CD15 (for the granulocyte assay) or only CD45 and CD64 (for the monocyte assay) (see Supporting Data, Figs. 9 and 10). Gated granulocytes, monocytes and lymphocytes appear as "PNH cells" in both assays and each of these leukocyte subsets should be clearly visible and on-scale in the FLAER versus CD24 plots (for the granulocyte assay) and FLAER versus CD14 plots (for the monocyte assay).

We recommend at a minimum, that the high-sensitivity RBC and granulocyte assays be run as a primary screen for samples submitted for PNH testing. It is recommended for both RBC and granulocyte assays that the reagents used be pre-mixed (cocktailed). Any samples found to contain PNH RBCs or granulocytes should thereafter be screened with the high-sensitivity monocyte assay. A recent comparison of over 450 PNH cases revealed that monocyte clones sizes were larger than the granulocyte clone sizes in approximately half of the samples. Interestingly, in cases where monocytes clones

were larger, they were often considerably larger than the granulocyte clones (AI, unpublished data). While the clinical significance or otherwise of this finding is uncertain without long-term follow up of the individual cases, these observations support the utility of assessing both lineages to more accurately determine the true PNH clone size in the WBCs. In support of this notion, other recent studies have found that monocyte and reticulocyte analyses often give the best estimation of clone size in patient samples with small clones (35).

Finally, it should be noted that Type II granulocytes and Type II monocytes can occasionally be detected in PNH WBC assays. Some reagents are better able to delineate Type II from Type III phenotypes (36). However, for reporting purposes it is important to include both Type II (when present) and Type III granulocytes and monocytes in the total PNH clone size reported. While the clinical significance of finding Type II WBC phenotypes is unknown at this time, it is recommended that their presence be reported alongside the total clone size as the clinical significance may be established at a later point.

At the current time, UK NEQAS is the only source of stabilized whole PNH blood available through quality assurance/proficiency testing schemes (27). While similar material from North American sources is currently not available through CAP (College of American Pathologists), such has become available recently through the QMP-LS (Quality Management Program – Laboratory Services) in Canada. Until similar material can be made available to QC/PT schemes in the United States, it may be difficult for many laboratories, especially those performing PNH testing infrequently, to have confidence in the design, reliability and sensitivity of their PNH flow testing methodologies and protocols. Until such controls become available, it is recommended that laboratories should share both positive and negative samples with other laboratories at defined intervals. It is also recommended that the reactivity of the antibodies/cocktails employed in PNH analysis should be verified (on a normal sample) within laboratory-defined intervals if a positive and negative target cell population is not encountered during this interval (38).

With the reagent sets used in this study, normal (non-PNH) populations of cells that are invariably present (even in cases with very large PNH clones) serve as both "internal positive" and "internal negative" controls for all the reagents used in the assays described herein. The use of internal controls, (reviewed in 39) which is recommended for monitoring the day-to-day performance of reagent sets in the leukemia/lymphoma immunophenotyping setting, is a very effective way to monitor antibody performance and verify optimal instrument setup for the three high-sensitivity PNH assays described here.

For the Red Cell assay, PNH RBCs should show bright expression of CD235a and even in PNH samples with very large clones, some normal Type I cells should be detectable to serve as a positive internal control to monitor CD59 conjugate performance. Reagent cocktailing is also highly recommended for the high-sensitivity RBC assay, so that if poor mixing of sample and antibodies occurs, and a subset of cells appear poorly stained, the latter are poorly stained with both CD235a and CD59, and will not be confused with PNH phenotypes as long as the operator does not rely upon single parameter histograms for analysis (see Supporting Data).

For the granulocyte assay, granulocytes serve as an internal positive control for CD15 while lymphocytes serve as an internal negative population. Even in PNH samples with very large granulocyte clones, some normal granulocytes (FLAER+, CD24+) should be detectable if sufficient cells are collected. Regardless of the ability to detect normal granulocytes in such a sample, FLAER is expressed on normal lymphocytes (internal positive control) even in samples from patients with longstanding disease who have PNH phenotypes among their lymphocytes. Similarly, CD24 is expressed on normal B-lymphocytes (internal positive control) but not on normal T-lymphocytes (internal negative control). Neither FLAER nor CD24 are expressed on PNH lymphocytes when the latter are present.

For the high sensitivity monocyte assay, monocytes serve as an internal positive control and lymphocytes serve as an internal negative control for CD64. Normal monocytes serve as an internal positive control and lymphocytes serve as an internal negative control for CD14. As for the granulocyte assay, lymphocytes serve as an internal positive control for FLAER.

Detection of PNH granulocytes, PNH monocytes, and PNH lymphocytes (when present) in both granulocyte and monocyte assays in the appropriate places demonstrates proper instrument set-up and compensation for all three major leukocyte subsets (Figs. 2 and 3). If all populations are not properly visible and "on-scale," overcompensation is the most likely explanation and adjustments should be made to remedy this situation (see Supporting Data).

CONCLUSION

This manuscript describes basic protocols for the detection of PNH clones, both in PNH disease and other syndromes that have been associated with such. We have consciously limited the analysis to two colors for RBC and four colors for WBC and suggest a tiered approach—a high resolution RBC and neutrophil assay followed by a reflex monocyte assay in cases where PNH cells are detected in either assay. While it is possible to combine antibodies toward neutrophils and monocytes in a high-sensitivity assay using six or more colors, it is our opinion that this approach is unnecessary for the routine clinical flow cytometry laboratory and will lead to increased cost and complexity with respect to instrument setup, compensation and selection of appropriate antibody conjugates. A further advantage of our approach is that the assays described herein can be performed across a range of instruments with four or more PMTs with minimal changes to the conjugate

combinations used. Research laboratories on the other hand may wish to increase the total number of markers to define specific subsets of cells showing absence of GPI-linked proteins.

It is hoped this simplified but robust approach using validated antibody clones and conjugates will facilitate the standardization of this assay. International quality assurance studies are currently planned to document the robustness of these assays across multiple sites.

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